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The Attachment of V79 and Human Periodontal Ligament Fibroblasts on Periodontally Involved Root Surfaces Following Treatment with EDTA, Citric Acid, or Tetracycline HCL: An SEM *in vitro* Study

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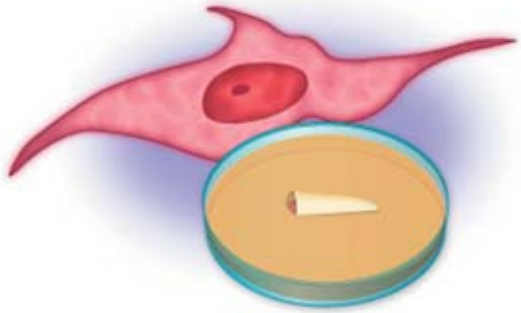

Abstract

Objective: The present *in vitro* study has been designed to establish and compare the effects of citric acid, EDTA, and tetracycline HCl on human periodontally diseased roots on the structure, attachment, and orientation of V79 (primary Chinese hamster lung fibroblasts) cells and human periodontal ligament fibroblasts (HPDL).

Materials and Methods: Commercially availableV79 cells and HPDL derived from healthy human third molars were used in this study. These fibroblasts were left in solution for seven days in order to attain confluence. Forty single-rooted teeth were obtained from patients diagnosed with periodontitis. The crown part was removed under constant irrigation and the root was split vertically into two equal halves, thus, yielding 80 specimens. Following scaling and root planing, the specimens were washed with phosphate buffered saline (PBS) and kept in 50 µg/ml gentamycin sulphate solution for 24 hours. The root pieces were then treated as follows: citric acid at pH 1, 24% EDTA, or with a 10% solution of tetracycline HCl and were then placed in V79 fibroblast cultures and HPDL cultures. The specimens were harvested after four weeks and were fixed in 2.5% glutaraldehyde in PBS before preparation for scanning electron microscopy (SEM).

Results: The behavior of V79 cells was similar to that of human periodontal ligament cells on root conditioned surfaces. V79 and HPDL showed a healthy morphology on root surfaces treated with citric acid and EDTA and a relatively unhealthy appearance on root surfaces treated with tetracycline HCl and distilled water (control group).

Conclusion: The results suggest the use of citric acid and EDTA as root conditioning agents favorably affects the migration, attachment, and morphology of fibroblasts on human root surfaces, which may play a significant role in periodontal healing and regeneration.

Keywords: Human periodontal ligament fibroblasts, HPDL, V79 cells, root-conditioning, EDTA, citric acid, tetracycline HCl, periodontal diseases

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Introduction

Root surfaces affected by periodontal diseases present with structural and chemical changes due to contamination by cytotoxic and other biologically active substances from periodontal pathogens.[1,2,3] Several studies have clearly demonstrated bacteria and their endotoxins can contaminate the root surfaces of periodontally affected teeth. In addition this bacterial contamination prevents the reattachment of gingival and periodontal cells. Such surfaces are not biocompatible with adjacent periodontal cells, the proliferation of which is pivotal for periodontal wound healing.[1,4] It is not possible to decontaminate periodontitis-affected root surfaces by mechanical means alone as hand or ultrasonic scaling of root surface produces a nonbiocompatible smear layer.[4,5,6]

Root conditioning agents have been used in surgical periodontal therapy mainly for two reasons. They aid in the dissolution of the smear layer after a relatively short exposure time and selectively remove root surface-associated mineral, thus, exposing the underlying collagen to varying degrees.[7] However, the ability of root-conditioning agents to aid in periodontal regeneration has been questioned in both experimental *in vitro* and *in vivo* studies.[1,8,9]

The ultimate goal of periodontal therapy is full regeneration of the periodontium destroyed by periodontitis.[10] From experiments *in vitro*, fibroblast migration, attachment, and orientation seem to be important steps in the formation of a new connective tissue attachment to the root surfaces. It is not unreasonable to assume, therefore, that following root conditioning similar behavior of fibroblasts is expected in the regenerative process.[11,12] Different root surface qualities are reported to influence fibroblast functions, e.g., endotoxins from periodontal pathogens may penetrate the root surface and have been implicated in inhibiting fibroblast proliferation, synthesis, and attachment. Root conditioning agents have been known to have an effect on the migration, attachment, and orientation of fibroblasts, thus, suggesting there is a greater chance of new connective tissue attachment to root surfaces which have been demineralized.[11,13]

A number of *in vivo* studies, however, report no additional advantage of surface demineralization over root planing at least in terms of flap healing. The inconsistency of these studies may be due to differences in experimental systems and techniques. For example, *in vivo* studies are frequently compared with *in vitro* studies. Surface demineralization of periodontally diseased roots has been compared with surface demineralization of non-diseased roots, often utilizing roots from different species as well as different demineralizing agents. Finally, different criteria for the assessment of wound healing have been used. There is also a paucity of studies which assess the effects of root conditioning on non-oral and non-human cell groups.[7,8,14,15]

The present study has been designed to establish and compare the effects of citric acid, EDTA, or tetracycline HCl on human periodontally diseased roots on the structure, attachment, and orientation of V79 (primary Chinese hamster lung fibroblasts) cells and human periodontal ligament fibroblasts (HPDL) *in vitro*. V79, also known as V79-HG04 fibroblast, is a transformed cell line of a primary culture derived from embryonic

lung fibroblasts of a Chinese hamster. This cell line was first established in 1959.[14] Its diameter is about 18 µm (range: 15-25 µm), and its morphology is described as "fibroblastoid" or "fibroblast-like adherent cells" and has a diploid karyotype. It is primarily used in toxicity and cell fusion studies and is devoid of infectious viruses or toxic products, thus, making it very safe to handle (Table 1). In culture it closely resembles other groups of fibroblasts and grows in clumps.[14] Requisite measurements were made at 2000X and 4000X magnifications, but varying magnifications were used to record the appropriate findings.

Materials and Methods

Materials

Forty periodontally involved human teeth scheduled for extraction were used in the study. The teeth were devoid of caries or restorations and were extracted for periodontal reasons only.

Culture of HPDL and V79 Cells

To evaluate whether the root surfaces were suitable for fibroblast reattachment or for *in vitro* regeneration, HPDL derived from healthy human third molars were used. The periodontal tissue was separated from the root surface with a sterile surgical blade. The tissue was collected, minced, treated with collagenase (0.1 units/ml), washed twice with phosphate buffered saline (PBS), and was then transplanted into 25 cm^2 tissue culture flasks (Nunc, Rosklide, Denmark) containing Modified Eagle's Medium (MEM) [Flow, Cat 14-100-54 UK] with 50 µg/ml of gentamycin sulphate, 1% L-glutamine (SIGMA), and 10% fetal calf serum (FCS). The transplants were then left for outgrowth. V79 cells were procured from the National Centre for Cell Sciences, Pune, India. These cells were grown in MEM supplemented with 10% FCS, 1% l-glutamine, and 50 µg/ml gentamycin sulfate. The cells were routinely cultured in 25 cm^2 flasks (Nunc, Rosklide, Denmark) with loosened caps, in 5% CO_2 in air in a humidified atmosphere at 37°C.

Primary cell cultures and cell lines were cultivated in MEM supplemented with gentamycin, L-glutamine, and 10% FCS at 37°C in 5% CO_2 with 0.2% EDTA (Kebob-Lab, Sweden) in PBS buffer and for 1 min with 0.1% crude trypsin (Flow, UK) in PBS. The contents of each flask were suspended in fresh growth medium and transferred to three new flasks. Primary cell cultures used for the test were in the third to eight passages.

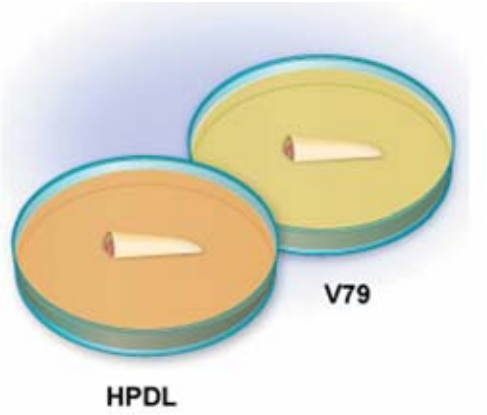
Preparation of the Teeth

Forty single-rooted teeth were obtained from 36 patients diagnosed with chronic periodontitis. The

Table 1. Properties of V79 cells.

V79 CELLS PROVIDE UNIQUE BIOLOGICAL RPOPERTIES:[14,34]
• They have a stable diploid karyotype as in human cells.
• The cloning efficiency is greater than 90%.
• Their doubling time is 12 hours, thus, the cells reach confluence in a shorter duration.
• They exhibit stable morphology with very few deviations from the normal structure.
THE FOLLOWING PROPERTIES MAKE THE V79 A UNIQUE *IN VITRO* TEST SYSTEM:[14,34]
• **Humanized** : Humanized cells guarantee high predicitivity of *in vitro* results for the human situation. V79 cells resemble human fibroblasts in morphology and in culture but are of smaller size.
• **Specific** : Specificity enables to clarify metabolic pathways, structure, functions as well as mechanisms in various comparative studies with other cell groups.
• **Standardized** : Standardized systems guarantee high reproducibility.
• **Easy to use** : V79 cells are stable and reproducible homogenous systems.
• **Economic** : Excellent cost/benefit ratio due to improved data quality with high predictive value for the human situation.

crown part was removed under constant irrigation, and the root was split vertically into two equal halves, thus, yielding 80 specimens. These specimens were thoroughly root planed with a Gracey curette until a smooth glass like surface was obtained and were then washed with PBS and were kept in 50 µg/ml gentamycin sulphate solution for 24 hours so as to disinfect them. They were then randomly divided into the following groups.



Group I: Control group of ten specimens treated with distilled water and placed in HPDL cultures.

Group II: Control group of ten specimens treated with distilled water and placed in V79 fibroblast cultures.

Group III: Ten specimens treated with 24% EDTA for two minutes and placed in HPDL cultures.

Group IV: Ten specimens treated with 24% EDTA for two minutes and placed in V79 fibroblast cultures.

Group V: Ten specimens treated with citric acid at pH 1 and placed in HPDL cultures. The solution was applied for five minutes to the experimental root surfaces with cotton pellets moistened with the agent. These solutions were applied with light pressure to allow the agent to wet the surface to burnish the agent into the root surface.

Group VI: Ten specimens treated with citric acid at pH 1 and placed in V79 fibroblast cultures.

Group VII: Ten specimens treated with tetracycline HCl solution prepared by dissolving a 500 mg capsule (Hostacycline ® , Aventis-Pasteur) in 5 ml of distilled water (10% solution) and placed in HPDL cultures. The solution was applied for five minutes with a light pressure to burnish the agent into the root surface.

Group VIII: Ten specimens treated with tetracycline HCl solution and placed in V79 fibroblast cultures.

Experimental Procedure

The above-mentioned fibroblasts were left in solution for seven days in order to attain confluence. The 80 specimens were divided as follows: 40 specimens were placed in the V79 fibroblast cultures, while the other 40 specimens were placed in HPDL cultures. One specimen was placed at the center of each petri dish. The specimens were harvested after four weeks and were fixed in 2.5% glutaraldehyde in PBS before preparation for scanning electron microscopy (SEM).

Scanning Electron Microscopy (SEM)

All the specimens were prepared for SEM in the following manner: the specimens were dehydrated in a graded series of ethanol and finally with 100% acetone as a final step. The specimens were mounted on aluminum stubs sputter coated with colloidal gold and examined using a SEM (Cambridge Stereoscan-120 SEM–Altran Corporation, Boston, MA, USA) operating at 20kv and at a tilt angle (Shallow Angle Technique).

Results

Group I: Control group specimens treated with distilled water and placed in HPDL cultures

The root surface presented with an irregular amorphous coating defined as the smear layer. Few dentinal tubules were visible or they were partially obscured. The root surface showed sparse distribution of cells. The average cell size was 17 µm. The cells appeared to have retracted from the root surface and have a roughly circular shape characteristic of unattached cells. The vitality of the cells appeared poor with the outer surface of the plasma membrane characterized by numerous oval shaped blebs, microridges, and foldings (Figure 1).

The blebs were uniform in size and distribution and probably represented pinocytotic or exocytotic vesicles or impressions of cytoplasmic organelles in the overlying plasma membrane. The cells were devoid of cytoplasmic extensions like lamellopodia. There was an increase in the cellular debris around the cells. There was an increase in the number of cells around the vicinity of dentinal tubules, but the cells did not exhibit lamellopodia in these sites because there was no collagen fiber exposure in the dentinal tubules as the root surface was not treated with root conditioning agents (Figure 2).

Group II: Control group specimens treated with distilled water and placed in V79 cultures

The root surface was covered with the smear layer and few open dentinal tubules were visible. The average cell size was 15 µm. At lower magnification, the cells were essentially flattened and were surrounded by cell debris and the smear layer. At higher magnification, the cells showed flattening but were devoid of lamellopodia and the vitality appeared poor. As in the HPDL cells of the control group, the outer surface of the plasma membrane was characterized by numerous oval shaped blebs, microridges, and foldings probably representing pinocytotic or exocytotic vesicles or impressions of cytoplasmic organelles in the overlying plasma membrane (Figure 3). There was an increase in the number of cells around the vicinity of dentinal tubules, but the cells did not exhibit lamellopodia inthese sites because there was no collagen fiber exposure in the dentinal tubules.

Group III: Specimens treated with 24% EDTA and placed in HPDL cultures

On EDTA treated specimens, the HPDL cell morphology was strikingly different. The major characteristic was the extreme flattening and spreading of the cells on the root surface making the contours of the cell hardly visible. Average cell size was between 18-22 µm. However, inspite of the extreme spreading, the cells exhibited thickened, short lamellopodia ranging from 2.25-4 µm. The cell surface was smooth and continued smoothly onto the lamellopodial extensions, which seem to be originating from one surface only (Figure 4).

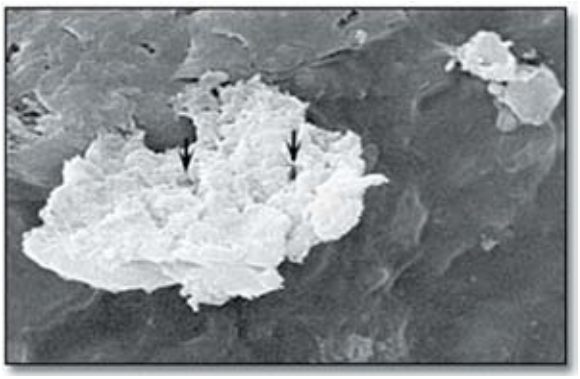


Figure 1. HPDL showing foldings and fenestrations on the plasma membrane (arrow). The irregular, amorphous coating on the root surface can also be seen *(Magnification X3800)*.

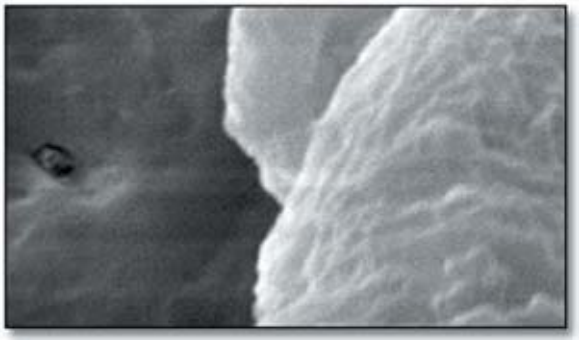


Figure 2. HPDL with a smooth margin devoid of lamellopodia in the vicinity of a dentinal tubule *(Magnification X10000)*.

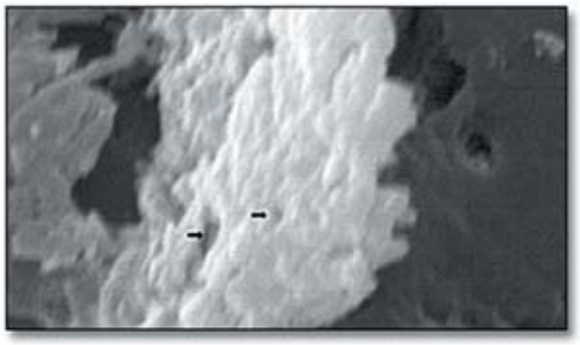


Figure 3. Terminal portion of V79 cells showing numerous oval shaped blebs, microridges, and foldings (arrow) on the plasma membrane *(Magnification X12000)*.

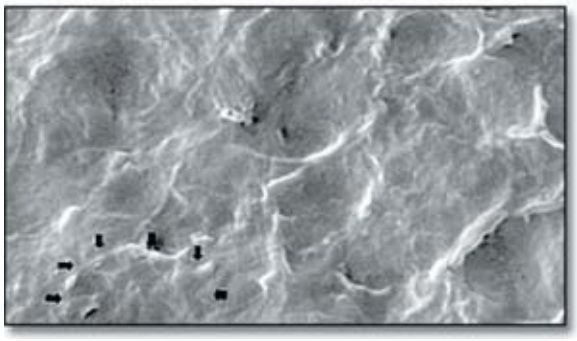


Figure 4. HPDL showing extreme flattening and spreading of the cells (arrow) in the root surface. Thickened, short lamellopodia can also be seen *(Magnification X3000)*.

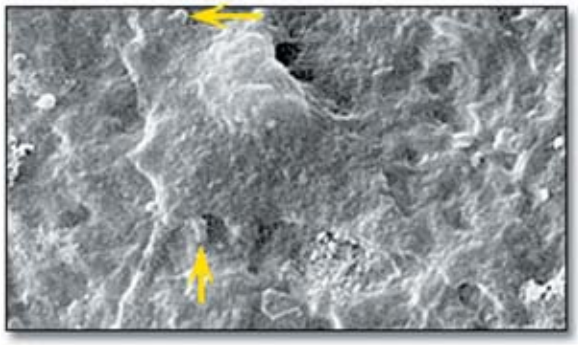


Figure 5. HPDL with a "fried egg" appearance showing developing and established lamellopodia. Angular areas can also be seen *(Magnification X3800)*.

Root surfaces having low cell numbers showed slightly different morphology. The cells showed a rounded elevation ("fried egg" morphology) in the centre caused by the nucleus which was 7 µm in diameter. This structure was absent in areas of high cell concentrations. Two to three lamellopodia usually developed from each cell and were of an average length of 8 µm and between 1.25-1.5 µm in width (Figure 5). In between the lamellopodia, angular areas probably leading to cell flattening were seen.

Group IV: Specimens treated with 24% EDTA and placed in V79 cultures

The V79 cells also exhibited the extreme flattening and spreading of the cells on the root surface obscuring the root surface (Figure 6). The average cell size was 14 µm. Cell surface was relatively smooth without blebs or ruffles. The cells exhibited small lamellopodia of 4 µm in length. The cell, however, seemed to be showing about five to six lamellopodia.

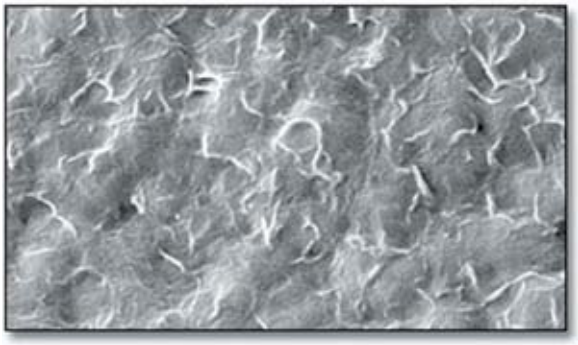


Figure 6. V79 cells exhibiting extreme flattening and spreading obscuring the root surface. Small numerous lamellopodia can be seen *(Magnification X2000)*.

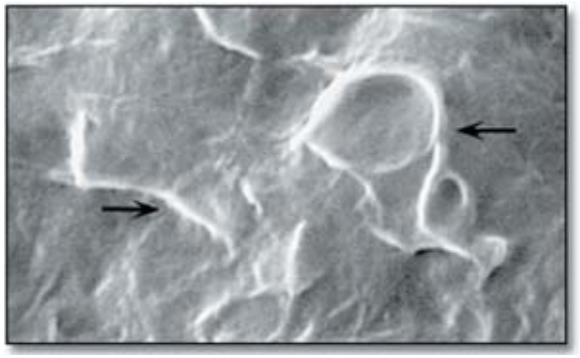


Figure 7. V79 cells with flattened cell surface and extending lamellopodia. The cells have a predominantly angular outline with areas progressing into gradual flattening *(Magnification X6000)*.

High magnification revealed a wholly flattened cell surface with extending lamellopodia. Rather than having a smooth outline, the cells had a predominantly angular outline with areas progressing into gradual flattening (Figure 7). These angular areas in between the lamellopodia were more prominent in the V79 group than in the HPDL group and may represent initial stages of cell flattening.

Group V: Specimens treated with citric acid at pH 1 and placed in HPDL cultures

At low magnification, areas with low fibroblast density shows a prominent exposed collagenous mesh work, which continues as a large sheet. The average cell size was 22 µm. The fibroblasts appeared tubular and were almost embedded in the mesh work (Figure 8).

Higher magnification reveals the structure of a healthy HPDL fibroblast. The lamellopodia were

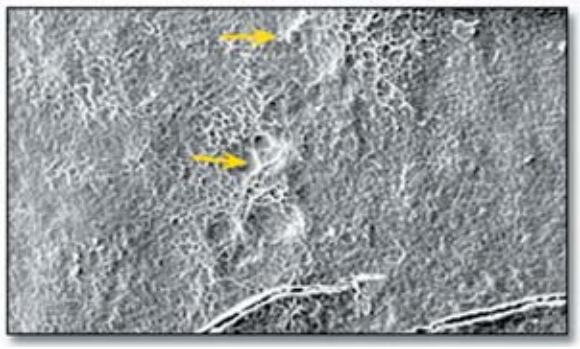


Figure 8. HPDL appearing tubular (arrow) and almost embedded in the meshwork *(Magnification X190)*.

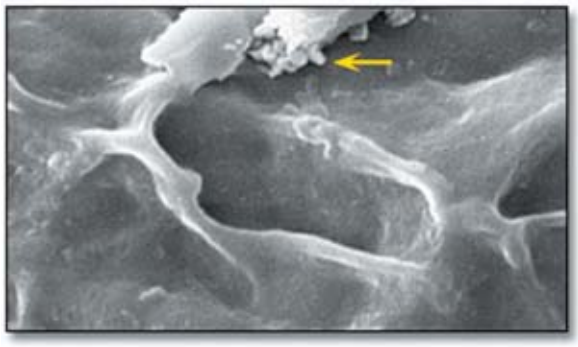


Figure 10. V79 showing multiple villous lamellopodia (arrow) *(Magnification X4000)*.

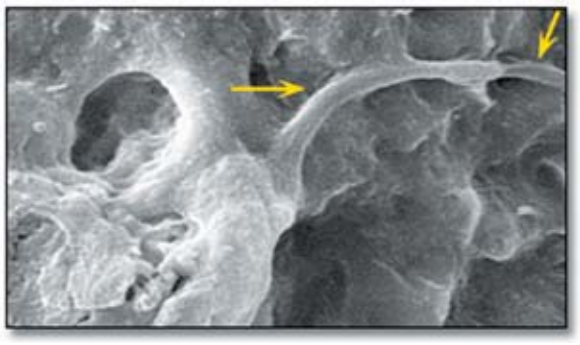


Figure 9. HPDL showing a "fried egg" appearance with thick lamellopodia (arrow) extending into the surrounding collagen meshwork (open arrow) caused by acid etching *(Magnification X4000)*.

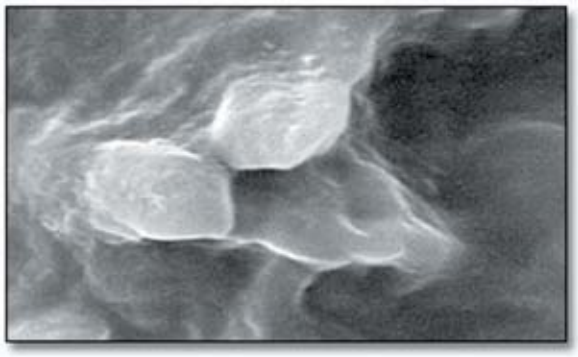


Figure 11. Multiple lamellopodia arising from a V79 cell *(Magnification X6000)*.

thicker, about 1-1.5 µm, and were longer, at about 13 µm, than in the EDTA specimens and seem to orient parallel to the exposed fibrils (Figure 9). The lamellopodia in these cells seem to arise very close from the cell body though this was not always the case. The lamellopodia generally extended into the areas of dentinal tubules and into the surrounding collagen meshwork caused by etching.

Group VI: Specimens treated with citric acid at pH 1 and placed in V79 cultures

The cells more or less resembled the HPDL cells. The average cell size was 18 µm. They did not exhibit the elevated area in the center as found in the HPDL. The cells exhibited multiple lamellopodia; usually more than seven in the vicinity of the exposed collagen fibers (Figure 10). These were characteristically "villous" and two or more lamellopodia arose from the same area. Lamellopodia were short at 0.5-1 µm in length with a thickness of 2.5 µm at its origin (Figure 11). The cell surface was relatively smooth and homogenous with a vital appearance.

Group VII: Specimens treated with tetracycline HCl solution and placed in HPDL cultures

The surface of the tetracycline treated areas showed a characteristic etching pattern at concentrated sites with a moderate amount of organic debris inside the etched areas. There was not much collagen fiber exposure as seen in citric acid and in EDTA, which reflected on the cell density. The average cell size was 20 µm. The cell structure varied from a moderately healthy to unhealthy appearance with an increased concentration of the cells at the well etched areas. The cells in these areas showed characteristic lamellopodia with lengths ranging from 0.75 µm to 4 µm. There were numerous small granules (about 1-2 µm in diameter) in the vicinity of these cells, which were presumed to be tetracycline granules and cells that were in higher number at their vicinity (Figure 12).

Group VIII: Specimens treated with tetracycline HCl solution and placed in V79 cultures

The surface of tetracycline treated areas showed characteristic etching pattern with distinct areas

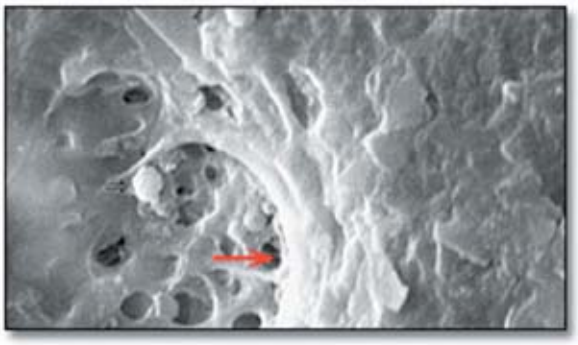


Figure 12. Spindle shaped HPDL with a solitary lamellopodia (arrow). The spherical granules are probably filler particles in Tetracycline HCL capsules *(Magnification X3200)*.

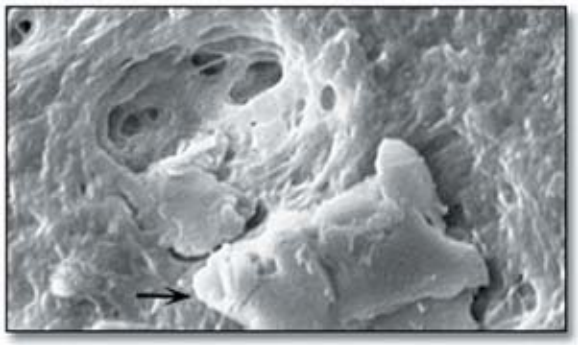


Figure 13. V79 cell with surface microridges and with a lamellopodia projecting away from the root surface *(Magnification X2000)*.

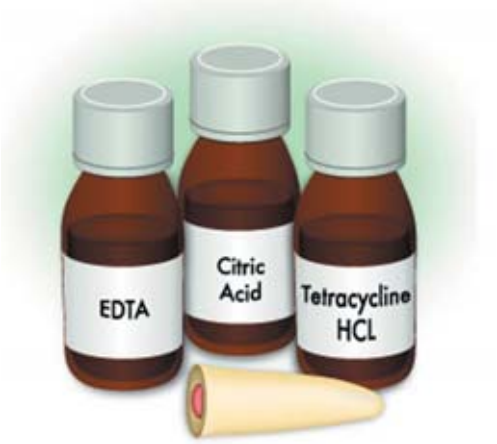
of patent dentinal tubules, and areas between the tubules exhibited a fibrillar matrix with a matted appearance. However, in some areas a smear layer still persisted. The average cell size was 20 µm. The majority of cells assumed a circular morphology characteristic of unattached cells. There is a low density of surface blebs and few, if any, lamellopodia or cytoplasmic extensions, and the cells have a detached appearance as in the control group. There seems to be an attempt to form lamellopodia, which were just 3 µm in length but had a hypertrophic, stubby appearance and did not seem intimately attached, as they appeared to project away from the root surface (Figure 13).

Discussion

Tetracyclines, citric acid, and EDTA are frequently used as root conditioners. They aid in the demineralization of root surfaces, eliminate the smear layer, aid in the opening of the dentinal tubules, and expose some components of the matrix like the type I collagen.[8] Several *in vitro* studies have suggested root surface modifications by citric acid, tetracycline HCl, and EDTA could influence the behavior of periodontal fibroblasts, improving their attachment and development.[2,10,13,16] Though there is no conclusive evidence regarding the benefits of root conditioning, it is being widely practiced in clinical management.[18]

The effect of citric acid at pH 1, 24% EDTA, and tetracycline HCl on the growth and morphology of non-human/non-oral cells, was evaluated. A HPDL cell is an appropriate cell line for evaluating cell attachment. It is relatively free of tissue remnants and shows minimal alteration of cell morphology and function when cultured.[14] V79 is a cell line used in cell attachment and toxicology studies. Very few studies exist regarding the effect of root conditioning on the growth and morphology on non-human/non-oral cells.[7,8,19,20] Wikesjo et al.[21] stated the sequence of healing events on dentinal surfaces implanted in bone cavities of beagle dogs conditioned with citric acid and Heparin was largely similar to healing at native dentinal surfaces. Lowenguth et al.[22] studied the morphology of the fiber attachment system *in vivo* by placing demineralized human dentine cylinders transcutaneously into the backs of rats and stated the cell and fiber orientation seen was structurally similar to a periodontal ligament. They stated the spatial approximation of appropriate substrates is essential for cell and fiber orientation. Baker et al.[9] developed a screening model for immediate evaluation of the influence of root condit-ioning protocols on the adsorption and adhesion of blood to the dentin surfaces. Planed and citric acid-treated human dentin surfaces were exposed to fresh blood, which was then allowed to clot before SEM evaluation. They stated the various preclinical systems employed were not robust enough to identify protocols of clinical relevance and stressed the importance of using other cellular systems, human and non-human, to understand vital events like wound healing.

The study protocol involved the placement of root planed specimens treated with Citric acid at pH 1, 24% EDTA, and tetracycline HCl (500 mg in 5 ml of distilled water) in HPDL and V79 cultures followed by an SEM examination to study the growth and morphology of V79 and HPDL fibroblasts.



The results suggest root conditioning improves the attachment and morphology of the fibroblasts. Both the V79 and HPDL fibroblasts in the control group that were root planed and treated with distilled water showed an unhealthy appearance with surface blebs, fenestrations, and microridges with the absence of cytoplasmic extensions like lamellopodia and were sparsely distributed on the root surface. The root surface exhibited the smear layer with no collagen exposure on the root surface, which may not be a compatible environment to the fibroblasts. Some specimens treated with tetracycline HCl also exhibited the smear layer and fibroblasts with surface microridges and fenestrations but with short stumpy lamellopodia, which appeared to project away from the root surface. This is in sharp contrast to the V79 and HPDL fibroblasts on the root-conditioned surfaces, which had a smooth glossy appearance and showed multiple lamellopodia, which interacted with the exposed collagen fibers on the root surface. This is in agreement with the observations of Fardal and Lowenberg[13] who stated root planing improved diseased roots by aiding in the orientation of fibroblasts towards root surfaces and acid demineralization subsequent to root planing, which exposed the underlying collagen fibers, thus, creating a more hospitable environment for fibroblast attachment. The results are also in agreement with those of Vanheusden et al.[23] who suggested conditioning of the root surface could promote the attachment, the proliferation, and the biosynthetic activity of periodontal ligament cells, which is an important prerequisite to periodontal regeneration. Most of the HPDL and V79 fibroblasts were found in the vicinity of dentinal tubules and the exposed collagen meshwork, more so in the groups treated with citric acid and EDTA than in the tetracycline HCl treated and untreated control groups. This observation is in agreement with those of Babay [7] who stated root conditioning and root planing "roughens" the root surface by collagen and dentinal tubule exposure, which may help the fibroblasts better attach to microscopic rough surfaces than to smooth-surfaced specimens.

From the morphological point of view, the influence of the three types of chemical conditioning was markedly different. The results showed treatment with citric acid and EDTA showed an extremely good fibroblast attachment, morphology, and architecture. The results obtained by EDTA and citric acid was in accordance with the results of various researchers[4,14,24,23], though Pant et al.[25] did not obtain a good result with EDTA as compared to other agents. Tetracycline HCl treatment showed a moderately healthy fibroblast morphology and attachment of both the HPDL and V79 fibroblasts, which also exhibited incipient lamellopodia. This is in agreement with the findings of Zaman et al[26] who stated EDTA and citric acid demineralization enhances HPDL attachment and orientation to the root surface to a greater degree than tetracycline HCL. Polson et al.[17], Polson and Proye[27], and Hanes et al.[28] have suggested this inconsistency may in part be explained by inadequate demineralization of the periodontitis-affected root, potentially by acid denaturation of the collagen matrix and by detrimental effect of acidic solution on migration of progenitor cells from the periodontal ligament. It has also been shown immersion of dentin specimens in a 0.5% TTC solution (pH 3.2) for five minutes may not completely remove the organic smear layer. The filopodia length and morphology was also unhealthy and is contrary to the findings of Rompen et al.[29] who stated tetracyclines might induce filopodia formation. Doubts have been raised about the use of tetracycline powder from commercial preparations as they might contain a significant amount of filler material.[30] We, however, used a commercially prepared capsule of tetracycline HCl (Hostacycline ®, Aventis-Pasteur) so as to simulate its use in an actual clinical situation.

HPDL fibroblasts showed a "fried egg'' morphology, i.e., a large centrally located nuclear bulge surrounded by a smooth circular

cytoplasmic region. The cells in these cases usually presented with a healthy appearance with a surface devoid of fenestrations and microridges and lamellopodia, which is in contrast to the observations of Payne et al.[19] who considered this appearance as a feature associated with extensive cellular damage leading to cell death. However, this was not the case in the present study, where cells showed healthy morphologic features.

HPDL and V79 cells showed good adaptation and growth on root conditioned surfaces and also showed developing and developed lamellopodia, which are essential for the initial migration and attachment of the fibroblasts. Rompen[31] observed root conditioning directly influenced the attachment properties of fibroblastic cells and various root-conditioning agents could act by different mechanisms to aid the fibroblasts. These structures "allow the cells to search for the substratum," and they are absent or reduced in number on cells that have attached to the substratum. These lamellopodia were found in root-conditioned specimens only and were absent in control group specimens. This is in contrast to the observation made by Al-Nazhan[7] who observed the smear layer on the root surfaces that are root planed, but not root conditioned, do not impede the attachment of the lamellopodia of the fibroblasts to the root surface.

In EDTA treated specimens, despite the extreme spreading and good attachment, both HPDL and V79 cells exhibited thickened, short lamellopodia. This is not in agreement with the observations of Van der Schueren et al.[32] who observed long cytoplasmic extensions are generally associated with cell locomotion and initial adhesion and are also absent or reduced in the number of cells that have attached to the substratum. It can be assumed the increased number of lamellopodia may have developed in an attempt to increase the ability of the cell to attach in the presence of any factor that was altering its ability to attach to the substratum, in this case the adjacent fibroblast.

In the EDTA and citric acid groups, V79 cells showed more lamellopodia than the HPDL fibroblasts. The average number of lamellopodia was four to seven for V79 cells and three to four for HPDL cells. V79 cells also exhibited more angular areas in between lamellopodia than HPDL, which probably represent areas of flattening of cells. The phases of exploration and flattening seem to be independent processes (Figure 14).

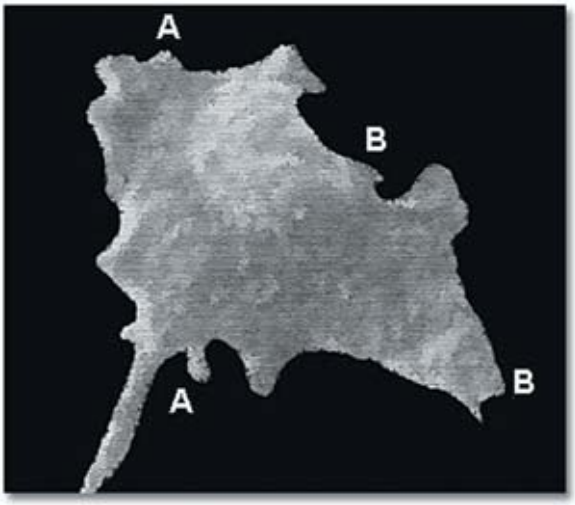


Figure 14. Illustration of a fibroblast based on the SEM findings showing areas with lamellopodia (A) and angular areas (B), which seem to contribute to cell spreading and flattening. This was observed in both V79 and HPDL.

This is not in agreement with the observations of Lowenberg et al.[33] and Al-Nazhan[7] who consider extension of filopodia as a precursor to cell flattening. This observation can be closely correlated to the findings of Payne et al.[19] who found well developed lamellopodia of HPDL on a highly foreign material like surgical grade calcium sulphate biomaterials than on a more biocompatible membrane like polylactic acid membrane (PLA). In the same analog V79 cells, which were grown on a foreign root surface like human root specimens, may have responded by more lamellopodia formation than HPDL fibroblasts.

As in any *in vitro* investigation, the obtained results cannot be extrapolated to an *in vivo* situation and often the results of *in vitro* studies are, at best, only indirectly related to the intact biologic system. However, the results suggest during periodontal surgery conditioning the root surface by citric acid, tetracycline, and EDTA could promote the proliferation and attachment of fibroblasts and, thus, aid in periodontal regeneration.

Conclusion

The aim of the present study was to establish and compare the effects of the application of citric acid, EDTA, and tetracycline HCl on human periodontally diseased roots on the structure, attachment, and orientation of V79 cells and HPDL *in vitro*. V79 cells are of smaller size but resemble human fibroblasts in morphology and in culture and guarantee high predicitivity of *in vitro* results for a human situation.[34]

The study demonstrated that:

- The behavior of V79 cells was similar to that of human periodontal ligament cells on root conditioned surfaces.
- V79 and HPDL on the root-conditioned surfaces had a smooth glossy appearance, showed multiple lamellopodia for locomotion, and in general showed angular areas between the lamellopodia, which appeared to be areas of progressive cell flattening.
- V79 and HPDL showed a healthy morphology on root surfaces treated with citric acid and EDTA and a relatively unhealthy appearance on root surfaces treated with tetracycline HCl and distilled water (control group).
- Finally, the observations suggest the use of citric acid and EDTA as root conditioning agents favourably affects the migration, attachment, and morphology of fibroblasts on human root surfaces, which may play a significant role in periodontal healing and regeneration. Tetracyclines, however, did not show results in the same lines as those of citric acid and EDTA.

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